Tsung-Han Stanley Hsieh / Tjian-Darzacq group at UC Berkeley.
Please contact hsieht@berkeley.edu if any question.
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MICRO-C FOR MAMMALIAN CELLS (2018-v0p99)

I. PREPARE CROSSLINKED CHROMATIN FROM CELL CULTURE

1. Culture cells in recommended condition.
2. Harvest cells by centrifugation for 5 min at 800xg at room temperature.
3. Resuspend cells in **base media (w/o FBS)** in a concentration of **1x10^6 cells/mL** (max. 30mL in 50mL tube).
4. Add **16% Formaldehyde** to a final concentration of **1%** (e.g. 2mL for 30mL sample).
   Incubate for 10 min at room temperature with mixing.
5. Add **2M Tris pH7.5** to a final concentration of **0.75M** to quench the reaction (e.g. 18.75mL for 30mL sample).
   Incubate for 5 min at room temperature.
   Centrifuge for 5 min at >800xg at room temperature. Discard supernatant.
6. Wash cells twice by 1X PBS in a concentration of 1x10^6 cells/mL.
   Centrifuge for 5 min at >800xg at room temperature. Discard supernatant.
7. Freshly prepare long crosslinkers solution as described in table:

Crosslinkers	MW	Spacer (Å)	Stock	Working
DSG	326.26	7.7	300mM in DMSO	**3.0mM in PBS**
EGS	456.36	16.1	300mM in DMSO	**3.0mM in PBS**

   Ex. 50mg DSG + 510.84 DMSO = 300mM DSG → +50mL PBS = 3mM DSG
8. Resuspend cell pellet in long crosslinker solution in a concentration of 1x10^6 cells/mL.
   Incubate for 45 min at room temperature with mixing.
9. Add **2M Tris pH7.5** to a final concentration of **0.75M** to quench the reaction.
   Incubate for 5 min at room temperature.
   Centrifuge for 5 min at >800xg at room temperature. Discard supernatant.
10. Wash cells twice by 1X PBS in a concentration of 1x10^6 cells/mL.
    Centrifuge for 5 min at >800xg at room temperature. Discard supernatant.
11. Snap freeze cell pellets by liquid nitrogen.

II. DIGEST CROSSLINKED CHROMATIN BY MICROCOCCAL NUCLEASE

1. Freshly complete **MB#1**:

Total	10 mL	5 mL	2.5 mL	Final
MB#1	9700 µL	4850 µL	2425 µL	50mM NaCl, 10mM Tris, 5mM MgCl2, 1mM CaCl2, 0.2% NP-40, 1X PIC
10% NP-40	200 µL	100 µL	50 µL	
100X PIC	100 µL	50 µL	25 µL	

2. Resuspend cell pellet in complete MB#1 in a concentration of 1x10^6 cells/100µL.
   Incubate for 20 min on ice.
   Centrifuge for 5 min at ~10000xg for at 4°C. Discard supernatant.
3. Wash nuclei pellet in complete MB#1 in a concentration of 1x10^6 cells/100µL.
   Centrifuge for 5 min at ~10000xg for at 4°C. Discard supernatant.
4. Resuspend nuclei pellet in complete MB#1 in a concentration of 1x10^6 cells/100µL.
5. Add appropriate amount of **MNase** to digest chromatin to **90% Monomer/10% Dimer**.
   Incubate for 10 min at 37°C with shaking at 850 rpm.
6. Add **500mM EGTA** in a final concentration at **4mM** to stop reaction.
   Incubate for 10 min at 65°C.
   Centrifuge for 5 min at >10,000xg at 4°C. Discard supernatant.
7. Wash/Rinse nuclei pellet in 1mL of cold **MB#2** twice.
   Centrifuge for 5 min at >10,000xg at 4°C. Discard supernatant.

III. REPAIR FRAGMENT ENDS

A. END-CHEWING			
Total	100 µL	50 µL	Final
Chromatin from 1-5 x 10^6 cells	pellet	pellet	50mM NaCl, 10mM Tris, 10mM MgCl2, 100µg/mL BSA 2mM ATP 5mM DTT
10X NEBuffer 2.1	10	5	
100mM ATP	2	1	
100mM DTT	5	2.5	
H2O	68	34	
10U/µL T4 PNK	5	2.5	~10U/1µg DNA (*NEB End repair module: 5U for 1ug DNA*)
→ Incubate for 15 min at 37°C.			
5U/µL Klenow Fragment	10	5	~10U/1µg DNA (*NEB End repair module: xxU for 1µg DNA*)
→ Incubate for 15 min at 37°C.			

B. END-LABELING			
Total	150 µL	75 µL	Final
Chromatin	100 µL	100 µL	**66mM dNTP / each** 33mM NaCl, 23mM Tris, 10mM MgCl2, 100µg/mL BSA 1.67mM ATP 6.67mM DTT
1mM Biotin-dATP	10	5	
1mM Biotin-dCTP	10	5	
10mM dTTP + dGTP	1	0.5	
10X T4 DNA Ligase Buffer	5	2.5	
20mg/mL BSA (200X)	0.25	0.125	
H2O	23.75	11.875	
→ Incubate for 45 min at 25°C with interval mixing. → Add **500mM EDTA** in a final concentration at **30mM** → 65°C for 20 min. → Centrifuge for 5 min at > 10,000xg at 4°C. Discard supernatant. → Rinse once with 1mL of cold **MB#3**. → Centrifuge for 5 min at > 10,000xg at 4°C. Discard supernatant.			

IV. PROXIMITY LIGATION & PURGE UNLIGATED ENDS

A. LIGATION			
Total	500 µL	250 µL	Final
Chromatin	pellet	pellet	50mM Tris, 10mM MgCl2, 1mM ATP, 10mM DTT 100µg/mL BSA
Water	422.5	211.25	
10X T4 DNA ligase buffer *w/ ATP*	50	25	
20mg/mL BSA (200X)	2.5	1.25	
400U/µL T4 DNA ligase	25	12.5	XXU/1µg DNA (*optimal: ?*)
→ Incubate for > 2.5 hours at room temperature with slow rotation. → Centrifuge for 5 min at >16000xg at 4°C. Discard supernatant.			

B. REMOVE BIOTIN-dNTP FROM UNLIGATED ENDS			
Total	200 µL	100 µL	Final
Chromatin	pellet	pellet	10mM Bis-Tris-Propane-HCl, 10mM MgCl2, 1mM DTT
10X NEBuffer#1	20	10	
Water	170	85	
100U/µL Exonuclease III	10	5	XXU/1µg DNA (*optimal: ?*)
→ Incubate at 37°C for 15 min with interval mixing.			

C. REVERSE CROSSLINKING			
Total	250 µL	125 µL	Final

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Chromatin	200 µL	100 µL	-
80X Proteinase K	25	12.5	2mg/mL Proteinase K = 2X
10% SDS solution	25	12.5	1X SDS
→ Incubate at 65°C for overnight.			

V. Dinucleosomal DNA Purification

1. **Phenol:Chloroform:Isoamylalcohol (PCI) extraction:**
   → Add 1X volume of PCI → Vortex for 20 sec → Spin for 10min at 19800xg at room temperature → Keep upper layer.
2. **Purify DNA by ethanol precipitation:**
   → Add 0.1X volume of sodium acetate and 2.5X volume of 100% ethanol → Incubate for >1 hr at -80°C → Spin for 15min at 19800xg at 4°C → Wash pellet by 75% ethanol → Spin for 5 min at 19800xg at 4°C → Air dry pellet for 10min at room temperature → Resuspend pellet in 50 µL of TE buffer (+1X RNase A) → Incubate for >30min at 37°C → ZymoClean.
3. **Size-selection for di-nucleosomal DNA:**
   → Separate **Monomer** and **Dimer** by running on **3.5% TAE / 3% TBE NuSieve agarose gel** → Cut band at size **250–400 bp (Avoid cutting < 200 bp to eliminate monomer)** → ZymoGel purification and resuspend DNA in 18 µL of elution buffer → Quantify DNA by Qubit.

VI Library Preparation

1. End-it

Total	50 µL	
Input DNA	34	
10X End-it buffer	5	
10X ATP	5	
10X dNTP	5	
End-it enzyme mix	1	
→ 45 min @ 25°C → 10 min @ 70°C		

2. Streptavidin Purification

a) Wash 5 µL beads/sample by 1X TBW.
Note: 10 ug/1uL beads can bind 200 ng dsDNA or 5 pmol ssDNA. Input DNA is usually ~2 – 5 ug DNA (20 – 100 pmol). 5 uL can bind up to 1ug dsDNA or 100 pmol ssDNA.
b) Resuspend in 150 µL of 2X BW.
c) Mix with 150 µL of DNA sample for 20min at room temperature.
d) Wash by 1X TBW @ 55°C w/ interval mixing **x2**.
e) Wash by 10mM Tris.
f) Resuspend in 25 µL of EB buffer.

3. End Repair / A-tailing

Total	30 µL	
Input DNA	25	
End Prep Reaction Buffer	3.5	
End Prep Enzyme Mix	1.5	
→ 30 min @ 20°C w/ interval mixing → 30 min @ 65°C		

4. Adpater Ligation

Total	46 µL	
Input DNA	30	
Adapter for Illumina	0.5	
Ligation Master Mix	15	
Ligation Enhancer	0.5	
→ 30 min @ 20°C w/ interval mixing		
USER enzyme	1.5	
→ 15 min @ 37°C w/ interval mixing		

5. Bead Wash

a) Wash by 1X TBW @ 55°C w/ interval mixing.
b) Wash by 10mM Tris.
c) Resuspend in 24 µL of EB buffer.

6. Minimum PCR & Purification

Total	50 µL	
Streptavidin beads w/ EB	24	
2X KAPA HiFi Hot Start Mix	25	
10µM PE1.0 primer	0.5	
10µM PE2.0 primer	0.5	

Denaturation	98°C	45sec
8-12 cycles	98°C	15sec
	60°C	30sec
	72°C	30sec
Extension	72°C	1min
	4°C	Hold

→ Check library size by agarose gel
→ 0.9X Ampure XP beads purification
→ Quantify library by Qubit

VII. Deep Sequencing by Illumina PE-50 or PE-100

VIII. Materials and Reagents

- **DSG (disuccinimidyl glutarate)** (ThermoFisher #20593)
- **EGS (ethylene glycol bis(succinimidyl succinate))** (ThermoFisher #21565)
- **16% Formaldehyde** (Fisher scientific #NC1040701)
- **2M Tris pH=7.5** (Sigma Aldrich #)
- **MBuffer#1:** 50mM NaCl, 10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1mM $CaCl_2$, , 0.2% NP-40, 1X PIC.
- **MBuffer#2:** 50mM NaCl, 10mM Tris-HCl pH 7.5, 10mM MgCl2
- **MBuffer#3:** 50mM Tris-HCl pH7.5, 10mM MgCl2
- **Micrococcal Nuclease** (Worthington Biochem #LS004798)
  Resuspended from lyophilized powder at 20 U/µl in Tris pH 7.4. Aliquot into tubes upon first use and freeze at –80°C.
- **0.5M EGTA** (Fisher scientific #)
- **0.5M EDTA** (Fisher scientific #)
- **T4 DNA Polymerase** (New England Biolabs #M0203)
- **DNA Polymerase I, Large (Klenow) Fragment** (New England Biolabs #M0210)
- **T4 Polynucleotide Kinase** (New England Biolabs #M0201)
- **T4 DNA Ligase** (New England Biolabs #M0202)
- **Exonuclease III (*E. coli*)** (New England Biolabs #M0206)
- **Biotin-14-dATP** (Jena Bioscience #NU-835-BIO14)
- **Biotin-11-dCTP** (Jena Bioscience #NU-809-BIOX)
- **20X Proteinase K solution** (Sigma Aldrich # 3115879001)
  TE with 20 mg/ml proteinase K and 50% glycerol
- **Elution buffer:** 10 mM Tris-HCl pH 7.6
- **TE buffer:** 10 mM Tris-HCl pH 8.0, 1 mM EDTA
- **End-It DNA End-Repair Kit** (Lucigen # ER81050)
- **Dynabeads® MyOne Streptavidin C1** (Life Technologies # 65001)
- **KAPA HiFi HotStart ReadyMix** (KAPA Biosystems # KK2601)
- **NEBNext Ultra II** (New England Biolabs #E7645)